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Endosymbiotic bacteria can directly manipulate their host's sex determination towards the production of female offspring

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A recent study in the lepidopteran *Ostrinia scapulalis* shows that endosymbionts can actively manipulate the sex determination mechanism of their host. *Wolbachia* bacteria alter the sex-specific splicing of the *doublesex* master switch gene. In ZZ males of this female heterogametic system, the female isoform of *doublesex* is produced in the presence of the bacteria. The effect is a lethal feminization of genotypic males. Curing of ZW females leads to males that die, indicating that the bacteria have an obligate role in proper sex determination and development of their host. Microbial intervention with host sex determination may be a driving force behind the evolutionary turnover of sex determination mechanisms.

Keywords:
doublesex; endosymbiont; feminization; heterogamety; sex determination

Introduction

Bacterial endosymbionts such as *Wolbachia*, *Cardinium*, *Rickettsia*, *Arsenophonus*, and *Spiroplasma* occur ubiquitously in insects and some other invertebrate groups such as isopods, spiders, and nematodes [1, 2]. They manipulate their host's reproduction in several ways, including male-killing, feminization, and parthenogenesis induction. As these actions are often disadvantageous for the host, the microbes may induce genetic changes in the host to escape or counteract their manipulation [3]. Although these various effects on hosts have been well documented in an array of species, the mechanistic details of how the microbes interfere with host reproduction are still poorly known. Some studies have found evidence to suggest that endosymbionts interfere directly with the genetic pathway of sex determination (see below). In a recent publication, Sugimoto and Ishikawa [4] show that *Wolbachia* bacteria change males into females in the moth *Ostrinia scapulalis*. They alter the sex-specific splicing of *doublesex*, the master switch gene in sex determination, from the male to the female form.

As in all lepidopteran insects, *O. scapulalis* has female heterogamety, meaning that females are the heterogametic sex (ZW) and males are homogametic (ZZ). Some strains are infected with *Wolbachia* bacteria that are transmitted via the egg cytoplasm and cause feminization of genotypic males. However, feminized on a ZZ genetic background is lethal. Surprisingly, the absence of *Wolbachia* in the opposite sex (ZW – i.e. female) causes lethality, as antibiotic curing of infected individuals demonstrates. That finding indicates that the host has evolved a dependence on the endosymbionts. The authors show that feminized ZZ individuals express the female specific splice form and that cured ZW individuals express the male specific splice form of *doublesex*. This means that the endosymbionts attempt to turn males into females, and that genetic females require the bacteria for proper sex determination.

The study of Sugimoto and Ishikawa [4] provides a number of novel viewpoints on how endosymbionts may exert their host manipulation. First, feminization is achieved through manipulation of the sex determination pathway upstream of *doublesex*, and hence occurs early during embryonic development. Second, the removal of the bacteria results in mas-

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culinization of the host, indicating that the bacteria are also required for normal sex determination and that the innate host sex determination mechanism has degraded. Third, both feminization of genotypic male (ZZ) individuals and masculinization of genotypic female (ZW) individuals is lethal. The feminization effect is therefore effectively a son-killer phenotype. Below I discuss the consequences and ramifications of these findings in more detail and consider possible molecular interactions between symbionts and the sex determination mechanism of their host.

How do microbes alter host reproduction?

Symbionts that influence the reproduction of their host are generally considered as parasitic, because they shift the host's reproduction towards the production of one sex. This is almost always the female because they are inherited via the egg's cytoplasm. How such reproductive parasites confer their parasitic action, in terms of the molecular and biochemical mechanisms, is a contemporary field of active research [3]. Some specific systems have been studied in detail, such as sonkilling by *Arsenophonus* in the wasp *Nasonia vitripennis*. This is accomplished by inhibition of the formation of maternal centrosomes in the developing eggs [5]. Another well-known case is *Wolbachia* induced feminization in the isopod *Armadillidium vulgaris*. This apparently occurs through prevention of androgenic gland development in infected males [6, 7]. This latter example appears to suggest that the manipulative microbes act on the sexual differentiation during early development, i.e. following the process of sex determination in the embryo. However, the *Ostrinia* study shows that feminization is achieved much earlier, namely by interfering with the sex determination switch gene that starts up the sex specific differentiation. The idea that symbionts can alter the host's sex determination mechanism is not new. *Wolbachia*, *Cardinium*, and *Rickettsia* have been found to cause parthenogenetic development. In many hymenopteran insects they cause diploidization of haploid eggs [8, 9]. Haploids develop into males and diploids into females due to haplodiploid reproduction in these insects. The microbial effect is therefore to change the hosts' chromosomal constitution that serves to start up the male or female sex determination pathway. The novelty of Sugimoto and Ishikawa's study [4] is that the *Wolbachia* bacteria can apparently manipulate the sex determination pathway at a level below the initial ZZ versus ZW chromosomal signal. To understand how this can be accomplished a closer look at the lepidopteran sex determination mechanism is required.

The sex determination pathway in insects is partially conserved

Sex determination in insects has been well studied in a number of groups [10–12]. The overall emerging picture is that sex is determined through action of a cascade of genes that regulate each other in a hierarchical fashion (Fig. 1). There is evolutionary conservation at the bottom of cascades but diversification towards the top [13]. A conserved pattern is the

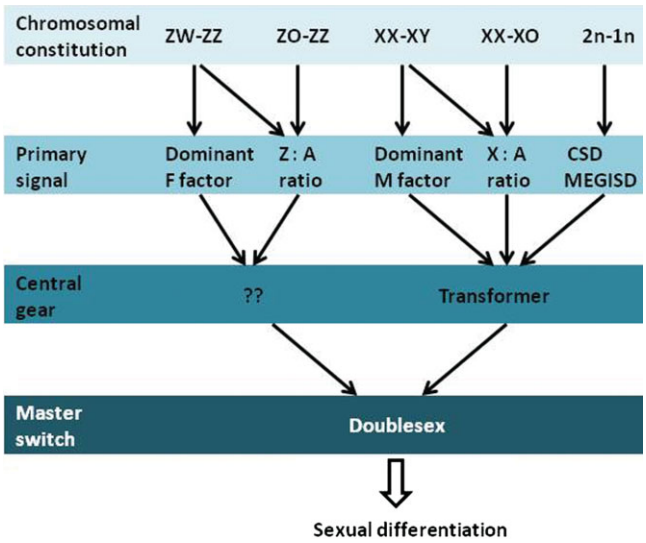

Figure 1. Levels of sex determination control at which symbionts may interfere with their host. The top level is the chromosomal constitution, which is either female heterogamety (female-male: ZW-ZZ or ZO-ZZ), male heterogamety (XX-XY or XX-XO), or haplodiploidy (2n-1n). One level lower is the primary signal, which can be a dominant sex determiner (F, feminizing factor; M, masculinizing factor), the sex chromosome to autosome ratio (Z:A or X:A), or a haplodiploid mode of sex determination (CSD, complementary sex determination; MEGISD, maternal effect genomic imprinting sex determination). The third level from above is the processing of the primary signal by the *transformer* gene (''central gear'') in most insects but still unknown in female heterogametic systems. The bottom level is the sex specific splicing of the *doublesex* gene that governs sexual differentiation.

transformer – *doublesex* axis [12]. The transformer gene can be considered as the central gear that mediates the primary signal towards alternative splicing of the *doublesex* gene, which is the master switch to start female or male specific development. The primary signals represent the actual genetic difference between males and females and serve to initiate the sexual differentiation pathway. Primary signals appear particularly diverse and range from dominant male determining genes to dominant female determining genes to X chromosome dose. Superimposed on this diversity is variation at the chromosomal level, such as male and female heterogamety and haplodiploidy.

Interestingly, sex determination in Lepidoptera seems to partially deviate from this general pattern, in that transformer is apparently not part of the cascade [14, 15]. In the silk moth *Bombyx mori*, the W chromosome is believed to carry a dominant female determiner (*F*-factor or *Fem*), which somehow regulates the P-element splicing inhibitor (PSI), which in turn induces male specific splicing of *doublesex*. Sugimoto and Ishikawa [4] suggest that the feminizing *Wolbachia* have taken over the role of the *F*-factor and prevent the male specific splicing of *doublesex*. However, the effect may also be exerted at the level of PSI or directly at *doublesex* splicing. Offspring lethality upon removal of the bacteria further indicates that the host's innate female determining gene has become mutationally silenced.

Microbes can directly intervene with host sex determination genes

The *Ostrinia* study encourages one to consider the possible ways in which microbes can directly intervene with their host's sex determination and how this could yield their manipulative phenotypes. For a good understanding of the scope of such sex determination manipulation it is useful to consider the different levels of regulation of host sex determination (Fig. 1). Microbial manipulation at the highest level would be to change the chromosomal constitution of the host embryo that serves as the trigger for the genetic cascade of genes to translate this information into male or female development. The abovementioned parthenogenesis inducing *Wolbachia* in haplodiploids is an example of such an alteration at the chromosomal level, because the bacteria change haploid males into diploid females. In diploid organisms, symbionts could conceivably also manipulate the chromosomal constitution of the embryo, for example by altering segregation of the sex chromosomes in the heterogametic sex. As males in XY systems do not transmit the bacteria, this is only expected in female heterogametic systems, such as Lepidoptera. To my knowledge, symbionts that increase segregation of W over Z chromosomes has, however, not yet been documented.

Alternative levels at which symbionts can directly interfere with the sex determination pathway are at the primary signal or below (Fig. 1). Symbionts could for instance directly target the alternative splicing of doublesex. This is for the time being a valid alternative to overtaking the *F*-factor (*Fem* gene) function to achieve feminization in *Ostrinia*. However, as there exist much more variation at the level of the primary signals, it is worth considering how symbionts can exploit this level of sex determination regulation. They could interfere with dominant female or male determining genes located on the sex chromosomes, or alter X or Z chromosome dosage relationships in systems that rely on X:A or Z:A ratios to start up the sex determination cascade. For example, the leafhopper *Ziginidia pullala* has ZO-ZZ sex determination and feminization by *Wolbachia* in this species is accompanied by alteration of gene expression [16]. It is conceivable that *Wolbachia* also affect the genes involved in Z chromosome counting.

Transformer is a candidate target gene for microbial manipulation

The *transformer* gene is considered as the spider in the web of insect sex determination as it can accommodate many different primary signals [12]. It is a prime candidate for manipulation by microbes for two reasons: (1) silencing of *transformer* by RNAi in females results in sex reversal, and (2) *transformer* is both maternally and zygotically expressed which makes the window of opportunity for manipulation large [17–22]. Studies in dipterans and hymenopterans have shown that *transformer* activity occurs through autoregulation. It is transcribed during oogenesis and maternally provided to eggs. *Transformer* transcripts in the egg initiate zygotic *transformer* transcription [17–19]. Both maternal and zygotic activity of transformer is required for female development. Male development occurs if the transformer autoregulatory loop is interrupted in the zygote. In diploids with male heterogamety, this is accomplished by the dominant male determiner on the Y chromosome, which is provided by the sperm.

As the intracellular endosymbionts are only transmitted maternally, microbial interference with *transformer* is expected to prevent autoregulation disruption. They could block the effect of masculinizers, such as the dominant male determiners (*M*-factors) on the Y chromosome. Unfortunately, although *M* factors are known from several dipteran species and are likely widespread in male heterogametic systems, they have not been cloned and characterized in any insect species yet. Without knowledge of the molecular action of *M* factors one can only speculate on how symbionts can prevent the masculinizing effect of *M* factors. Possible scenarios include inhibition of *M* products at the transcriptional or translational level, or, if *M* is a DNA binding site, sequestering of the particular product that blocks *transformer* autoregulation. Interestingly, in the housefly *Musca domestica*, a variant of the transformer gene is known that is insensitive to *M* factors and causes carrier zygotes to always develop into females [17]. Although the details of its molecular regulation are not yet known, this example shows that transformer action can be manipulated towards female production.

Other evidence for microbial manipulation of sex determination

Two additional studies to the *Ostrinia* one have found evidence to suggest that symbionts can directly interfere with host sex determination (Table 1). Giorgini et al. [23] showed that removal of *Cardinium* in the parthenogenetic wasp *Encarsia hispida* resulted in reversal of diploid females into diploid males rather than haploid males, which is commonly observed in curing studies of haplodiploids [8]. Hence, in this case the bacteria are not responsible for genome duplication but feminize diploid offspring. It implies that parthenogenetic reproduction is encoded by the host, but that the *Cardinium* bacteria are responsible for sex determination. Weeks et al. [24] found that curing of *Cardinium* in the asexual haploid mite *Brevipalpis phoenicis* changes haploid daughters into haploid

Table 1. Studies that have found evidence for direct manipulation of host sex determination by symbionts.

species	sex determination mechanism	symbiont	effect	references
Ostrinia scapulalis (Lepidoptera)	Female hetero-gamety (ZW-ZZ)	*Wolbachia*	Feminization of ZZ males	[4]
Encarsia hispida (Hymenoptera)	Haplodiploidy (1n-2n)	*Cardinium*	Feminization of diploids	[23]
Brevipalpis phoenicis (Acari)	Haploidy (1n)	*Cardinium*	Feminization of haploids	[24]

sons, which also suggests that feminization is accomplished through altering the host's sex determination.

A final feature of Sugimoto and Ishikawa's study that requires further explanation is the observation that both feminization of ZZ individuals and masculinization of cured ZW individuals is lethal. The authors hypothesize that the lethality that is associated with the sex change is due to a disruption of dosage compensation. As the extent to which dosage compensation occurs in Lepidoptera is currently debated [25], it remains to be seen whether this explanation is valid. Interestingly, Veneti et al. [26] showed that male killing by *Spiroplasma poulsonii* in *Drosophila melanogaster* depends on the presence of a functional dosage compensation complex. Taken together, these two studies suggest that feminization and male killing could converge onto a similar mechanism of dosage compensation disruption in female heterogametic systems.

Conclusions

Although evidence for direct targeting of invertebrate sex determination pathways by symbionts is still scarce, there appears to be ample scope for manipulation. The demonstration of a shift in *doublesex* sex specific splicing by feminizing *Wolbachia* in *O. scapulalis* [4] is the best evidence to date. More demonstrations are expected in the near future, in particular in taxa with female heterogamety. I have considered a number of potential levels at which symbionts may seize upon the sex determination cascade of their host. It is clear that much more research on the molecular regulation of sex determination is needed to fully grasp the scope of endosymbiont manipulation. A not yet mentioned, but more direct way of taking over sex determination, would be symbionts that acquire host sex determination genes by lateral transfer. The exchange of genes between symbionts and host are well established, but the transfer appears to be predominantly from symbiont to host rather than vice versa (references in [27]). Hence, even though it is not unthinkable that symbionts can carry sex determination genes, this still needs to be demonstrated.

Evolutionary theory predicts that hosts will not apathetically undergo these microbial manipulations of their reproduction. If symbionts can alter the regulation of sex determination genes, their hosts are expected to evolve compensatory mechanisms to regain control. This may lead to changes in the regulation of sex determination genes or even the recruitment of new genes into the sex determination pathway. This is exactly what is seen when comparing sex determination cascades between closely related insect taxa; they have high turnover rates. Symbionts may therefore drive evolutionary changes in their host sex determination mechanisms, which may help to explain the enormous diversity of sex determination mechanisms observed among invertebrates. *Wolbachia* have recently been applied to control disease vectors, such as the mosquito *Aedes aegypti* [28]. Whether this is going to lead to evolutionary changes in the sex determination mechanism of this species remains to be seen.

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